Geometric, Osmotic, and Membrane Mechanical Properties of Density-Separated Human Red Cells

By Otwin Linderkamp and Herbert J. Meiselman

Although there is evidence that the deformability of the entire red blood cell (RBC) decreases during aging, reports on changes in relevant specific properties associated with the aging process are limited and not in total agreement. The purpose of this study was to evaluate some of the factors that might contribute to this decreased deformability. Geometric, osmotic, and membrane mechanical properties of unfractionated, top ("young") and bottom ("old") RBC from 5 healthy adult donors were measured using micropipette techniques. Surface area, volume, and diameter of RBC were measured at osmolalities of 297, 254, 202, and 153 mosm/kg. Two membrane mechanical properties, surface shear modulus of elasticity ($\mu$) and time constant ($\tau_s$) of viscoelastic recovery, were studied only in isotonic media. At each of the osmolalities, volume and surface area of the bottom cells were about 25% lower than those of the top cells. Bottom cells showed smaller increases in volume with decreasing osmolality than top cells: the surface area remained constant with changing osmolality for all three groups. The surface area-to-volume ratio and the minimum cylindrical diameter of the bottom cells were essentially identical to the top cells. However, both the surface area index (actual area of RBC divided by area of a sphere of same volume) and the swelling index (maximal volume divided by actual volume) of the bottom cells were significantly lower than top RBC. The shear modulus of elasticity ($\mu$) was about 0.006 dyne/cm in all 3 RBC populations, indicating that the forces necessary to deform a portion of the membrane did not change with RBC aging. The viscoelastic time constant ($\tau_s$) was 0.148 ± 0.020 (SD) sec for the bottom RBC and 0.099 ± 0.017 sec for the top cells. This difference indicates that shape recovery following membrane deformation is delayed in old RBC. The membrane surface viscosity ($\eta$), calculated as the product of $\tau_s$ times $\mu$ was 0.95 ± 0.22 × 10^{-2} dyne-sec/cm for the bottom cells and 0.54 ± 0.15 × 10^{-2} for the top RBC. These data indicate that the relative deficit in membrane surface area and the increased membrane viscosity of old RBC may be important determinants for their decreased deformability and their eventual removal from the circulation.

During in vivo aging, human red blood cells (RBC) lose membrane fragments and water, leading to a decrease in volume and an increase in density.1,2 Age-dependent decreases in RBC deformability have been demonstrated by increased viscosity,3 impaired filterability,4 decreased elongation under high shear,5,6 and increased pressure required to aspirate entire RBC into micropipettes.7,8 Because the spleen functions as a small-pore filter that requires marked deformation of passing RBC,9 it has been suggested that the rigidity of old RBC contributes to their final destruction. However, it is still unclear what causes the decreased deformability of aging RBC.

The deformability of RBC is mainly determined by the membrane surface-area-to-volume ratio, the cellular morphology, the mechanical properties of the cell membrane, and the viscosity of the cellular contents.10 RBC need excess membrane surface area, beyond that required to enclose the cell volume, to deform and to adopt various shapes during their passage through the microcirculation. A membrane-bound sphere possesses the minimal surface area necessary to enclose its volume and therefore is relatively rigid since deformation can only occur via increased membrane surface area. The surface area and volume of RBC, fractionated according to their density, have been measured microscopically by Canham11 and by Nash and Wyard12 using a micropipette method. Neither study reported significant differences in the area-to-volume ratio between top ("young") and bottom ("old") RBC. More specific information can be obtained by considering the relative excess membrane surface area that can be expressed by the surface area index (actual area of RBC divided by area of a sphere of same volume) or by the swelling index (maximal volume divided by actual volume). At a constant surface-area-to-volume ratio, both the surface area index and the swelling index can be calculated to decrease with decreasing RBC size, thus indicating a decreased relative excess membrane surface area.

With age, RBC also demonstrate increased osmotic fragility.13 This increased fragility of old RBC could be explained either by a decrease in the relative excess membrane area or by increased water influx in hypotonic solution. The effect of hypotonic solutions on geometric properties of RBC with different age has not been studied yet.

The membrane mechanical properties of RBC can be assessed by determination of the resistance to extensional shear deformation at constant surface area (surface elastic shear modulus, $\mu$)14 and measurement...
of the recovery time after release of extended RBC (viscoelastic time constant, \( t_\varepsilon \)). The product of \( \mu \) times \( t_\varepsilon \) is the surface viscosity of the RBC membrane. The extent of cell deformation at steady state is largely determined by the shear modulus, whereas the membrane viscosity, via \( t_\varepsilon \), influences the rate at which the cell can assume a new shape. The membrane surface elastic shear modulus (\( \mu \)) of age-separated RBC has been measured by two groups. Heusinkveld et al.\(^{16} \) found \( \mu \) of old cells about twice as high as unfractionated RBC, while Nash and Wyard\(^{17} \) did not find significant differences. Measurements of other membrane mechanical properties, including the viscoelastic time constant, do not appear to exist for age-separated cells.

The present study was designed to determine selected geometric, osmotic, and membrane mechanical properties of density-separated human RBC. Measured geometric properties included cell surface area, cell diameter, and cellular volume; minimum cylindrical diameter, mean RBC thickness, surface area index, and swelling index were calculated from these measured data. The surface-area-to-volume ratio and the minimum cylindrical diameter of the bottom cells were essentially identical to the top cells, whereas both the surface area index and the swelling index of the bottom cells were significantly lower. Osmotic studies involved the measurement of the above-mentioned geometric properties in various hypotonic media; bottom cells showed smaller increases in volume with decreasing osmolality than top RBC. Membrane mechanical measurements included shear modulus of elasticity and the viscoelastic time constant, with membrane viscosity calculated as the product of these two properties. No differences were found for the shear modulus of elasticity, while the viscoelastic time constant, and thus the calculated membrane viscosity, were significantly greater for the bottom cells. These data suggest that geometric factors as well as membrane mechanical behavior may contribute to the finite lifespan of the circulating red cell.

### MATERIALS AND METHODS

#### Blood and RBC Preparation

Blood was collected from 5 healthy adult laboratory personnel via venipuncture into heparin (5 IU/ml) on 2 different occasions, once to study geometric properties and a second time to study mechanical properties of RBC. All measurements were made at room temperature (22 ± 1°C) within 4 hr after collection. Part of each blood sample was separated into 2 fractions using a technique similar to that described in other studies.\(^{14} \) Whole blood was placed into 4 mm internal diameter by 47 mm long plastic tubes and centrifuged for 15 min at 12,000 g. After centrifugation, each tube was cut at the border between the buffy coat and the RBC, and the top RBC layer was carefully removed and diluted into phosphate-buffered saline (PBS). The tube was cut again very close to the lower end and the bottom fraction was removed and diluted into PBS. Top and bottom fractions contained less than 5% of the least and most dense RBC, respectively. Whole blood (i.e., unfractionated cells) was also diluted into PBS. The PBS solution contained 0.030 M \( \text{KH}_2\text{PO}_4 \), \( \text{Na}_2\text{HPO}_4 \), plus 1 g/liter human serum albumin and had a pH of 7.40 ± 0.02 at 25°C. All RBC dilutions were made to a final packed cell volume of 0.1%. The osmolalities of the PBS solutions were 297, 254, 202, and 153 mosm/kg for the measurements of geometric properties and 297 mosm/kg for the measurements of mechanical properties of RBC. All solutions were filtered through 0.45 \( \mu \)m Millipore filters immediately prior to use. Native plasma was taken after centrifugation of a portion of each blood sample at 2000 g. The plasma was centrifuged a second time at 20,000 g for 15 min to remove platelets.

#### Micropipette System

Micropipettes were made from microfilament glass capillaries (A.M. Systems, Everett, Washington) using a pipette puller (D. Kopf Instr., Tujunga, California, Model 700C). Flat pipette tips were produced by breaking the tip against the edge of a microscope slide while under microscopic observation. The micropipette was mounted in a pneumatic micromanipulator (de Fonbrune, Orion Research, Inc., Cambridge, Massachusetts) and connected to a water-filled reservoir. Zero and aspiration pressures applied to the micropipette tip were adjusted by moving the reservoir vertically using a precision micrometer, thus allowing 0.01 mm H\(_2\)O pressure resolution. The dilute RBC suspension under study was placed into a special microchamber formed by a standard microscope slide and coverslip separated by a 1.0-mm thick, "U"-shaped stainless steel spacer.\(^{18} \)

A microscope-video system was employed for the RBC measurements. Its basic components included: (1) Leitz Ortholux II microscope with 40X, NA = 0.70 objective; (2) high resolution video camera (Cohu Co., San Diego, California, Model 4410); (3) video tape recorder (Sanyo Co., Compton, California, Model 7100) with slow motion and still frame capability; (4) video time generator (Odetics Co., Anaheim, California, Model 77), which provides 1/60 sec time resolution corresponding to the framing rate of the recorder; (5) high resolution video monitor (Conrac Co., Covina, California, Model SNA14); and (6) video micrometer (Bacon Instrument Co., Pasadena, California, Model 201), which displayed movable vertical and horizontal lines on the monitor screen and also displayed a number proportional to their separation. The video micrometer was calibrated with a standard microscope micrometer, and the overall geometric distortion of the entire system was less than 1%.

#### Geometric Properties of RBC

Surface area and volume of individual RBC were measured using micropipette methods similar to those of Jay and Canham\(^{19} \) and Nash Wyard.\(^{20} \) Micropipettes with an internal diameter of 1.9–2.1 \( \mu \)m were filled with plasma of the donor, then flushed with the albuminized PBS used to suspend the cells (i.e., 297, 254, 202, or 153 mosm/kg). This procedure prevents RBC adhesion to the glass walls of the pipette. After the pressure in the pipette was brought to zero, a RBC was aspirated by adjusting the pressure to −15 mm H\(_2\)O. One portion of the cell entered the pipette assuming a cylindrical shape, while the outer part became spherical. The surface area (A) and volume (V) were calculated from the diameter of the pipette (D\(_p\)) and the length of the cell projection in the pipette (L) and the diameter of the outer portion (D\(_b\)):

\[
A = \pi(D_p + D_b^2 - D_b^4/4) \quad (1)
\]

\[
V = \pi/24 (6L^2D_b^2 + 4D_b^4 - D_b^6) \quad (2)
\]
These formulas do not consider the segment of the outer sphere, which is "cut off" by the tip of the pipette. This results in an error of less than 1% for surface area and volume. The diameter (D) of each RBC was measured before the cells were aspirated. The following parameters were calculated from the surface area (A), volume (V), and diameter (D) of the RBC: \[ A = \frac{4V^2}{\pi D^2} \] \[ V = \frac{4}{3} \pi \left( \frac{D^3}{12} \right) \] \[ \text{Surface area index} = \frac{A}{(4.84V^{2/3})} \] \[ \text{Swelling index} = 0.094A^{1/3}/V \] Minimum cylindrical diameter (Dc): \[ V = \left( \frac{ADc}{4} \right) - \left( \frac{\pi D^2}{12} \right) \] Note that the surface area index, which is the reciprocal of the sphericity index, has a limiting value of 1.0 for a sphere. The swelling index also has a limiting value of 1.0 for a spherical shape. The use of the surface area index, rather than the sphericity index, swelling index also has a limiting value of 1.0 for a spherical shape.

**Membrane Mechanical Properties**

Viscoelastic recovery time and surface elastic shear modulus of the RBC membrane were measured using techniques presented elsewhere.\[16\] Micropipettes with an internal tip diameter of 0.9–1.1 μm were filled with the donor's plasma diluted 1:10 with isotonic PBS. The dilute RBC suspension in albuminized isotonic PBS was introduced into the chamber. The chamber was inverted and the cells allowed to settle and attach to the lower glass surface for a period of 20 min. The 1:10 diluted autologous, platelet-free plasma was then used to gently flush the chamber until 2–3 chamber volumes had been displaced; this PBS-plasma solution aids in preventing further cell attachment. The chamber was then turned right-side up (i.e., RBC attached to inner surface of top coverslip) and placed on the stage of the microscope.

The membrane viscoelastic recovery time (t)\[15\] was determined by measuring the time-dependent shape recovery of individual RBC that had been extended by pulling at diametrically opposite points of the rim of the cell. The micropipette was positioned near the rim of the RBC to be measured, the water reservoir lowered slightly to produce a small "negative" pressure, and a hemisphere of the cell membrane aspirated into the tip of the pipette. Following verification that the RBC was only attached to the glass surface by a single point (i.e., "point-attached" cells) and that the pipette was diametrically opposite to this attachment point, the pipette was withdrawn until the cell attained an overall extension ratio of approximately 1.6. The pressure in the pipette was then rapidly returned towards zero and the cell allowed to pull free and recover its initial shape. Postexperimental measurements of cell length and width for each video field (i.e., every 1/60 sec), after the cell had begun to return to its initial shape, over a period of 0.33 sec (20 fields), provided the data required for computation of the viscoelastic recovery time. The recovery follows a time-dependent exponential behavior, and the viscoelastic time constant was calculated via a computer program from the individual measurements of length (L) and width (W), the first data point after releasing the cell (Lm, Wm), and the data 0.33 sec (20 fields) after releasing the cell (Lt, Wt): \[ e^{-\lambda t} = \frac{(L/W) - (L/W)m}{(L/W)m + (L/W)o} \cdot \frac{(L/W)m + (L/W)o}{(L/W) + (L/W)o} \cdot \frac{(L/W)m - (L/W)o}{(L/W)m - (L/W)o} \] The correlation coefficient was above 0.98 in each of the regressions calculated for single RBC.

The elastic shear modulus (μ)\[14\] was measured after the extended RBC had recovered its initial shape. The tip of the micropipette was positioned close to the central concave portion ("dimple") of the RBC. The pressure was adjusted to zero and then increased in 1 mm H2O steps to 5 mm H2O. This caused a "tongue" of the membrane to extend into the pipette. The surface elastic shear modulus (μ) was calculated from the applied pressure (P), the pipette diameter (Dp), and the lengths (L) of the cell projections in the pipette using the following linear relationship: \[ P = \frac{\mu (L/Dp)}{(2.45 L/Dp - 0.3015)} \] This approach is valid provided that no membrane folding or "buckling" occurs. When membrane buckling does occur, the cell folds and moves easily into the pipette (the fold is observable). Aspiration experiments in which membrane buckling or folding were observed were not used for shear modulus calculations.

The membrane surface viscosity (η) was calculated from the elastic shear modulus (μ) and the time constant (t) measured for each cell: \[ \eta = \mu t \] **Statistical Analysis**

Mean values and standard deviations were calculated for the 200 RBC (40 cells from each of the 5 donors studied in each experiment). The mean values of the top and bottom cells were compared using an unpaired two-tailed t test with 8 degrees of freedom.\[25\] The method of "cluster" sampling (each donor is one "cluster") is justified when it is reasonable to assume that the sample becomes more representative compared to the use of one cluster only (i.e., 200 cells from one donor) or of n clusters (one cell from each of 200 donors).\[25\] The latter would be difficult to achieve and would increase the influence of technical errors and the error introduced by personal preference of certain RBC.\[25\] However, the number of degrees of freedom used for statistical analysis should be based on the number of clusters rather than on the total number of observations because of possible interindividual differences.\[25\] Mean values and standard deviations were also calculated for the RBC parameters of each donor and showed no significant differences among the 5 donors (analysis of variance; Bartlett test).\[25\] The MCV values of the top and bottom RBC fractions were compared using a paired-difference t test. Linear regressions were calculated using the method of least squares.

**RESULTS**

The effects of decreasing osmolality on RBC geometric properties are shown in Table 1. At each of the osmolalities studied, the volume, surface area, and diameter of the bottom ("old") RBC were significantly lower than those of the top ("young") cells (p < 0.05); the values for the unfractoned RBC population lay between these density-separated groups. RBC volume
increased steadily with decreasing osmolality while the surface area remained constant. The diameter changed little as osmolality was lowered to 202 mosm/kg, whereas further reduction of the osmolality to 153 mosm/kg resulted in a marked decrease of the diameter.

The surface-area-to-volume ratio and the minimum cylindrical diameter of the bottom RBC were essentially identical to that for both the unfractionated and top cells over the entire range of osmolalities. The surface area index and the swelling index of the bottom RBC were essentially identical to that for both the unfractionated and experimental cell populations used in this study. The coefficient $A$ for the bottom cells was found to be significantly smaller than that obtained for the top cells ($p < 0.005$), whereas the reverse was observed for the intercept $B$. The $A$ and $B$ terms for the unfractionated cell populations were between those computed for the bottom and top cells.

Geometric and membrane mechanical properties of density-separated RBC in isotonic PBS are shown in Table 3. There were no significant differences between the RBC populations for the following parameters: (1) mean RBC thickness calculated from cell diameter and volume data; (2) membrane shear modulus of elasticity ($\mu$). In addition, RBC volumes measured by the electronic orifice system (MCV) and by the micropipette technique ($V$) did not show meaningful differences. The measured viscoelastic time constant ($t_e$) for the electronic orifice system (MCV) and by the micropipette technique ($V$) did not show meaningful differences. The measured viscoelastic time constant ($t_e$) for the electronic orifice system (MCV) and by the micropipette technique ($V$) did not show meaningful differences. The measured viscoelastic time constant ($t_e$) for the electronic orifice system (MCV) and by the micropipette technique ($V$) did not show meaningful differences. The measured viscoelastic time constant ($t_e$) for the electronic orifice system (MCV) and by the micropipette technique ($V$) did not show meaningful differences. The measured viscoelastic time constant ($t_e$) for the electronic orifice system (MCV) and by the micropipette technique ($V$) did not show meaningful differences.

### Table 1. The Effect of Different Osmolalities on Geometric Properties of RBC*

<table>
<thead>
<tr>
<th>Osmolality (mosm/kg)</th>
<th>Volume (fl)</th>
<th>Surface Area (µm²)</th>
<th>Diameter (µm)</th>
<th>Area/Volume</th>
<th>Surface Area Index</th>
<th>Swelling Index</th>
<th>MCD (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>297</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>U 89.8 ± 12.7</td>
<td>134.1 ± 13.8</td>
<td>7.88 ± 0.67</td>
<td>1.50 ± 0.05</td>
<td>1.39 ± 0.09</td>
<td>1.62 ± 0.07</td>
<td>2.86 ± 0.26</td>
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</tr>
<tr>
<td>T 98.1 ± 12.9</td>
<td>148.1 ± 15.1</td>
<td>8.50 ± 0.69</td>
<td>1.50 ± 0.08</td>
<td>1.45 ± 0.07</td>
<td>1.72 ± 0.09</td>
<td>2.81 ± 0.22</td>
<td></td>
</tr>
<tr>
<td>B 78.0 ± 11.8†</td>
<td>117.5 ± 14.6</td>
<td>7.40 ± 0.54†</td>
<td>1.51 ± 0.06</td>
<td>1.34 ± 0.08†</td>
<td>1.55 ± 0.06†</td>
<td>2.88 ± 0.20</td>
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<td>254</td>
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<tr>
<td>U 98.7 ± 13.2</td>
<td>132.4 ± 17.3</td>
<td>7.81 ± 0.65</td>
<td>1.34 ± 0.06</td>
<td>1.28 ± 0.06</td>
<td>1.45 ± 0.08</td>
<td>3.26 ± 0.28</td>
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<tr>
<td>T 109.1 ± 13.1</td>
<td>146.8 ± 13.2</td>
<td>8.50 ± 0.68</td>
<td>1.35 ± 0.06</td>
<td>1.33 ± 0.07</td>
<td>1.53 ± 0.07</td>
<td>3.20 ± 0.27</td>
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<tr>
<td>B 85.7 ± 12.2†</td>
<td>117.3 ± 12.7†</td>
<td>7.46 ± 0.72†</td>
<td>1.37 ± 0.06</td>
<td>1.23 ± 0.06†</td>
<td>1.39 ± 0.07†</td>
<td>3.25 ± 0.24</td>
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<tr>
<td>U 114.3 ± 17.8</td>
<td>133.4 ± 15.7</td>
<td>7.58 ± 0.55</td>
<td>1.16 ± 0.05</td>
<td>1.15 ± 0.07</td>
<td>1.26 ± 0.06</td>
<td>3.88 ± 0.35</td>
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<tr>
<td>T 127.8 ± 13.8</td>
<td>147.1 ± 11.8</td>
<td>8.25 ± 0.70</td>
<td>1.16 ± 0.05</td>
<td>1.21 ± 0.06</td>
<td>1.29 ± 0.06</td>
<td>3.85 ± 0.31</td>
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<tr>
<td>B 98.5 ± 13.4†</td>
<td>116.5 ± 14.9†</td>
<td>7.24 ± 0.61†</td>
<td>1.18 ± 0.07</td>
<td>1.12 ± 0.06†</td>
<td>1.19 ± 0.07†</td>
<td>3.96 ± 0.37</td>
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<td>153</td>
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<tr>
<td>U 137.7 ± 17.5</td>
<td>135.9 ± 14.7</td>
<td>6.92 ± 0.44</td>
<td>0.99 ± 0.04</td>
<td>1.06 ± 0.06</td>
<td>1.08 ± 0.07</td>
<td>5.01 ± 0.41</td>
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<tr>
<td>T 153.2 ± 23.2</td>
<td>151.7 ± 18.9</td>
<td>7.70 ± 0.78</td>
<td>0.99 ± 0.04</td>
<td>1.10 ± 0.06</td>
<td>1.15 ± 0.06</td>
<td>4.77 ± 0.33</td>
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<tr>
<td>B 116.3 ± 18.4†</td>
<td>118.4 ± 16.1†</td>
<td>6.49 ± 0.46†</td>
<td>1.02 ± 0.05</td>
<td>1.03 ± 0.06</td>
<td>1.04 ± 0.05†</td>
<td>5.12 ± 0.39</td>
<td></td>
</tr>
</tbody>
</table>

*Values represent mean ± one standard deviation calculated from 200 RBC (40 from each of 5 donors).

†p < 0.05 when compared to top RBC.

‡p < 0.01 when compared to top RBC.

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### Table 2. Relationships Between Changes in Osmolality and RBC Volume

<table>
<thead>
<tr>
<th>Source</th>
<th>Regression Equation*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sawitz et al.‡</td>
<td>V/V∞ = 0.572 (T/T∞) + 0.428</td>
</tr>
<tr>
<td>Evans and Fung‡</td>
<td>V/V∞ = 0.575 (T/T∞) + 0.431</td>
</tr>
<tr>
<td>Meiselman§</td>
<td>V/V∞ = 0.577 (T/T∞) + 0.436</td>
</tr>
<tr>
<td>Present report</td>
<td>V/V∞ = 0.565 (T/T∞) + 0.437</td>
</tr>
<tr>
<td>Unfractionated</td>
<td>V/V∞ = 0.595 (T/T∞) + 0.414</td>
</tr>
<tr>
<td>Top</td>
<td>V/V∞ = 0.520 (T/T∞) + 0.488</td>
</tr>
<tr>
<td>Bottom</td>
<td>V/V∞ = 0.520 (T/T∞) + 0.488</td>
</tr>
</tbody>
</table>

*V and V∞ are the mean RBC volumes at experimental (T) and isotonic osmolalities (T∞).

†MCV calculated as ratio of microhematocrit and RBC count.

‡RBC volumes determined microscopically.

§Unpublished data. MCV calculated as ratio of microhematocrit and RBC count.
was significantly larger for the bottom RBC when compared to the top cells \((p < 0.01)\), with the mean \(t_s\) value for the unfractionated cells lying between these two extremes. Thus, the calculated membrane viscosity \((\eta)\), computed as the product of \(\mu\) times \(t_s\), was significantly larger for the bottom RBC when compared to the top cells. Note that since \(\mu\) and \(t_s\) were measured independently for each RBC, it was possible to determine whether correlations existed between these two nominally independent parameters; no significant correlation was found between these two measures of membrane mechanical behavior for the three populations of cells \((r < 0.10)\).

**DISCUSSION**

The micropipette technique for determination of the surface area and volume of individual RBC requires comment due to possible sources of measurement errors:

1. Diffraction effects within the optical system limit the accuracy of linear measurements to about 0.25–0.50 \(\mu\)m. In addition, optical distortion caused by the glass micropipette makes an exact determination of the pipette inside diameter somewhat difficult.27 However, these possible absolute measurement errors appear to be of relatively minor importance in this study, inasmuch as the same pipette was used for all cells from the same donor and since the micropipette measurements of RBC volumes \((V)\) are in good agreement with volumes independently determined via the electronic orifice-type system (MCV, see Table 3). Thus, systematic errors do not seem to be responsible for the significant differences shown in Table 1.

2. At aspiration pressures of more than 100 mm H\(_2\)O, the cell projection in the pipette can lose one-third of its volume, while the surface area remains constant. The volume loss increases linearly with the applied pressure and with the portion of the RBC in the pipette.28 In the present study, we applied a pressure of only 15 mm H\(_2\)O and the portion of the RBC aspirated into the pipette was less than 40% at an osmolality of 297 mosm/kg and less than 20% at 153 mosm/kg. Thus, the calculated volume loss in our experiments was less than 2% at any osmolality.

3. At aspiration pressures below 10 mm H\(_2\)O, the outer portion of the RBC might not become completely spherical, resulting in overestimation of the volume and surface area.12 The use of 15 mm H\(_2\)O aspiration pressure in this study resulted in a spherical shape for the outer portion of the cell.

4. In pipettes greater than 2.5 \(\mu\)m internal diameter, the RBC may fold as it enters the pipette tip. Pipettes of 1.9–2.1 \(\mu\)m were employed for the present investigation, thus avoiding this possible artifact.

Both the volume and surface area of the top RBC were about 25% higher than the bottom cells (Table 1), but the surface-area-to-volume ratio of the top and bottom cells were not different. These surface-area-to-volume ratio determinations are in agreement with the results of Canham11 and Nash and Wyard,12 and thus contradict the general view that the surface-area-to-volume ratio to RBC decreases with age. However, the surface area and swelling indices indicate that the average top RBC has more excess surface area and more swelling ability than the average bottom cell. This suggests that old RBC have a relative deficit of surface area that could explain the increased osmotic fragility13 of old RBC and might contribute to their decreased deformability.18

Osmotic swelling of RBC is characterized by increasing cellular volume and thickness, decreasing cell diameter, and constant membrane surface area.27–29 Area dilatation requires very large forces, and the surface area of the membrane can increase by less than 3% before lysis occurs.28 The regression equations shown in Table 2 indicate that the bottom cells are less responsive to hypotonic media than the top cells, and that the results obtained in the present study for unfractionated RBC are consistent with other literature reports.27,28 Using the regression equations, it can be estimated that the fraction of osmotically active intracellular factors is about 56% in
The lower fraction in old cells is most likely a result of the higher hemoglobin concentration (MCHC)\textsuperscript{29} in the older cells. It can also be calculated from the surface area data in Table 1 and the regression equations in Table 2 that the mean osmolality at which RBC reach their spherical ("critical") volume is 138 mosm/kg for the unfractionated, 129 mosm/kg for the top, and 146 mosm/kg for the bottom RBC. Thus, old cells swell less with decreasing osmolality than young RBC but nevertheless hemolyze at a higher osmolality due to their lower swelling index.

Our study of mechanical properties of aging RBC did not reveal significant differences of the membrane elastic shear modulus (\(\mu\)) between different RBC populations (Table 3), confirming the findings of Nash and Wyard.\textsuperscript{37} The normal elastic shear modulus of old RBC thus indicates that the resistance of their membrane to shear deformation is normal.\textsuperscript{14,18} However, the viscoelastic time constant (\(t_c\)) and the calculated membrane surface viscosity were significantly increased in the bottom cells. The time constant depends on the viscous dissipation within the membrane and in the hemoglobin solution after release of the extended RBC.\textsuperscript{15} In normal cells, the mechanical power dissipated inside the RBC is less than 1% of the energy dissipated in the membrane. The dissipation inside the cell depends on the internal viscosity,\textsuperscript{15} and it can be calculated that the viscosity of hemoglobin in RBC increases from 9 to 54 cP during aging due to the increased MCHC.\textsuperscript{30} However, this increase in viscosity raises the contribution of mechanical power dissipated inside the RBC to only about 6%. Therefore, in old RBC, the time constant is still mainly the resultant of membrane elastic and membrane viscous forces.

The membrane fragments lost during RBC aging consist mainly of lipids, since the amount of membrane lipids decreases with age, while neither the amount nor the composition of membrane proteins change markedly.\textsuperscript{8,31} Spin label studies, which measure the motion of molecules incorporated into the membrane, show decreased lipid fluidity and decreased motion of membrane proteins in old RBC.\textsuperscript{8,32} These findings suggest that alterations in lipid–protein interaction play an important role in the aging process of the RBC membrane.\textsuperscript{32} The relationships between the motion of molecules in the membrane and the membrane viscous properties measured mechanically still remain unclear. However, both surface diffusivity and surface viscosity of the RBC membrane are probably influenced by dissipative processes in the membrane.\textsuperscript{15} Thus, the spin label studies might also indicate increased membrane surface viscosity in old RBC.

What role could the alterations of geometric and mechanical properties play in the destruction of old RBC? The most likely site of RBC membrane loss and eventual removal is the spleen, where the cells are transitorily trapped and concentrated.\textsuperscript{33} A normal RBC passes the spleen about 3600 times during its life, with a mean residence time of 1.4 min/transit.\textsuperscript{9} It is unknown how frequently the RBC has to pass through the approximately 1.5 by 5 \(\mu m\) measuring slits in the sinus walls, but it is clear that each passage requires marked deformation. Both decreased excess surface area and increased membrane viscosity of old RBC could contribute to their decreased cellular deformability.\textsuperscript{10} Decreased excess surface area diminishes the ability of RBC to adopt various shapes during their passage through the microcirculation. However, the low excess surface area of the old RBC does not increase their minimum cylindrical diameter because the old cells are markedly smaller than the young RBC (Table 1). This suggests that old RBC could pass capillaries and pores of the same size that young cells can pass. The decreased swelling index of old RBC indicates that they hemolyze at a higher osmolality than young cells. Under normal conditions, low osmolality does not appear to occur in any circulatory compartment. Moreover, the pH in the spleen and other organs is not sufficiently acid\textsuperscript{34} to promote swelling of RBC.\textsuperscript{35} Thus, it appears possible that neither the decreased excess surface area nor the low swelling capacity of aging RBC contribute to their final destruction.

The increased membrane viscosity of old RBC, however, most likely results in slower rates of cellular deformation, since under dynamic conditions, the red cell acts as a viscoelastic body.\textsuperscript{14,15} That is, the force required for a given deformation is dependent on the rate of deformation as well as the final deformed state. Thus, it would take more time for an old cell to assume different shapes during its passage through the microcirculation. This slower rate of deformation would therefore tend to delay the older RBC entry time into small vessels as well as their passage time through small pores. Thus, the old RBC probably require more time to pass the narrow splenic slits, thereby aiding in the recognition and destruction of these effete cells by macrophages.\textsuperscript{36,37} Note that the viscoelastic time constant for the older cells is about 50% greater than for the younger cells (Table 3) and that the magnitude of these time constants is consistent with the calculated residence times for RBC in mammalian capillaries.\textsuperscript{38} This correspondence between the time constants and the residence times suggests that the altered dynamic mechanical behavior of the old RBC may also be an important determinant of their flow behavior in the general microcirculation.
PROPERTIES OF SEPARATED HUMAN RBC

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Geometric, osmotic, and membrane mechanical properties of density-separated human red cells

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